ELISA PRODUCT INFORMATION & MANUAL

TUNEL Assay Kit – BrdU-Red

NBP2-54863

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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NBP2-54863 TUNEL Assay Kit – BrdU-Red

For a convenient and sensitive method to detect DNA fragmentation by flow cytometry and fluorescence microscopy in live cells.
This product is for research use only and is not intended for diagnostic use.

Materials Supplied and Storage

Store components of kit at -20°C or 4°C in the dark immediately upon receipt, according to storage conditions described in Materials Supplied section. Kit has a storage time of 1 year from receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components below.

Aliquot components in working volumes before storing at the recommended temperature.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Positive Control Cells	5 mL	-20ºC	-20ºC
Negative Control Cells	5 mL	-20ºC	-20ºC
Wash Buffer	120 mL	4ºC	4ºC
Reaction Buffer	0.6 mL	4ºC	4ºC
TdT Enzyme	45 µL	-20ºC	-20ºC
Br-dUTP	0.48 mL	-20ºC	-20ºC
Rinse Buffer	120 mL	4ºC	4ºC
Anti-BrdU (red) antibody	0.3 mL	4ºC	4ºC
7-AAD/RNase Staining Buffer	30 mL	4ºC	4ºC

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to perform this assay:

- Flow cytometer capable of detecting fluorescence at Ex/Em =488/576 nm (BrdU-Red) and Ex/Em = 488/655 nm (7-AAD)
- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Paraformaldehyde solution
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 12 x 75 mm test tubes designed for flow cytometer
- General tissue culture supplies
- 70% Ethanol

For IHC-P protocol:

- Fluorescence microscopy capable of measuring fluorescence at Ex/Em =488/576 nm (BrdU-Red) and Ex/Em = 488/655 nm (7-AAD)
- Coplin jars
- Glass slides
- Xylene
- Ethanol in the following percentages: 100% - 95% - 85% - 70% - 50%
- Coverslips (plastic and glass)
- 0.85% NaCl solution
- 10 mg/ml Proteinase K
- 100 mM Tris-HCl, pH 8.0 + 50mM EDTA solution
- (Optional) Anti-fading mounting solution - we recommend Fluoroshield Mounting Medium (ab104136)
- (Optional) Nail polish or rubber cement

1. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

1.1 Positive Control Cells (HL-60 cells treated with camptothecin): Ready to use as supplied. Thaw cells on ice. Take 1 mL aliquot (1 x 106 cells/mL) to use as positive control in the assay. Aliquot the rest of the cells in 1 mL aliquots and store at -20°C.

1.2 Negative Control Cells (HL-60 cells): Ready to use as supplied. Thaw cells on ice. Take 1 mL aliquot (1 x 106 cells/mL) to use as positive control in the assay. Aliquot the rest of the cells in 1 mL aliquots and store at -20°C.

1.3 Wash Buffer: Ready to use as supplied. Equilibrate to RT before use. Store at 4°C.

1.4 Reaction Buffer: Ready to use as supplied. Equilibrate to RT before use. Store at 4°C.

1.5 TdT Enzyme: Ready to use as supplied. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C.

1.6 Br-dUTP: Ready to use as supplied. Aliquot Br-dUTP so that you have enough volume to perform the desired number of assays. Store at -20°C.

1.7 Rinse Buffer: Ready to use as supplied. Equilibrate to RT before use. Store at 4°C.

1.8 Anti-BrdU antibody: Ready to use as supplied. Equilibrate to RT before use. Store at 4°C.

1.9 7-AAD/RNase Buffer: Ready to use as supplied. Equilibrate to RT before use. Store at 4°C.

2. Assay Procedure – Flow Cytometry

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Each cell line should be evaluated individually to determine the optimal cell density.
- Control cells included in the kit are already fixed. You can start using them from Step 2.3.2. Thaw cells on ice.

2.1 Grow and treat cells:

2.1.1 Grow cells (adherent or suspension) and induce apoptosis by your desired method.

Δ Note: Concurrently incubate a control culture without induction.

2.1.2 Collect 1-5 x 10^6 cells and resuspend in 0.5 mL PBS.

2.1.2.1 Suspension cells: collect cells by centrifugation.

2.1.2.2 Adherent cells: trypsinize cells in trypsin/EDTA for 1-2 min, stop trypsinization by adding culture medium and pellet by centrifugation.

Δ Note: take cell supernatant from adherent cell culture and centrifuge together with trypsinized cells.

2.2 Cell fixation:

2.2.1 Fix the cells by adding 5 mL of 4% paraformaldehyde (w/v).

2.2.2 Place cells on ice for 15 minutes.

2.2.3 Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.

2.2.4 Wash cells in 5 mL of ice-cold PBS. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.

2.2.5 Repeat washing step one time.

2.2.6 Re-suspend cells in 0.5 mL PBS

2.2.7 Add cells to 5 mL of ice-cold 70% (v/v) ethanol. Let cells stand for 30 minutes – overnight on ice (or at -20°C).

2.3 Label cells:

2.3.1 Resuspend fixed cells by swirling the vials.

2.3.2 Remove 1 mL aliquots of the cell suspension at 10^6 cells/mL and place 12 x 75 mm tubes.

Δ Note: use this procedure for control cells included in the kit.

2.3.3 Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and aspirate ethanol.

2.3.4 Resuspend cells in 1 mL Wash Buffer. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and aspirate supernatant carefully.

2.3.5 Repeat washing step one time.

2.3.6 Resuspend cells in 50 µL of the DNA Labeling Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
TdT Reaction Buffer	10	100
TdT Enzyme	0.75	7.5
Br-dUTP	8	80
ddH_2O	32.25	322.5
TOTAL VOLUME	51	510

2.3.7 Incubate cells in DNA labeling solution for 60 minutes at 37°C. Shake cells every 15 minutes to resuspend.

2.3.8 Add 1 mL of Rinse Buffer to each tube. Centrifuge for 5 minutes at 300 xg in a cold centrifuge and aspirate supernatant.

2.3.9 Repeat rinsing step one more time.

2.3.10 Resuspend cells in 100 µL of Antibody Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
Anti-BrdU-Red antibody	5	50
Rinse Buffer	95	950

2.3.11 Incubate cells in the Antibody Solution in the dark for 30 minutes at room temperature.

2.4 Detection by flow cytometry:

2.4.1 Add 500 µL of 7-AAD/RNase A solution.

2.4.2 Incubate cells in the dark for 30 minutes at room temperature.

2.4.3 Analyze cells by flow cytometry: Ex/Em = 488/576 nm for BrdU-Red and Ex/Em = 488/655 nm for 7-AAD.

Δ Note: analyze cells within 3 hours of staining.

ALTERNATIVE PROTOCOL FOR GFP-TRANSFECTED CELLS

Ethanol will affect the GFP signal. To detect DNA fragmentation in GFP-expressing cells, we recommend the following alternative protocol:

- Proceed with general protocol until Step 2.1.2
- Fix cell by adding 5 mL of 1% paraformaldehyde/PBS and incubate on ice 15 minutes
- Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Wash cells in 5 mL of ice-cold PBS. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Repeat washing step one time.
- Wash cells in 5 mL of ice-cold PBS.
- Resuspend cells in 2 mL of the following detergent solution:
  PBS solution (pH 7.2-7.4)
  0.1% Triton X-100
  4% FBS
- Incubate cells in detergent solution 3-5 minutes at room temperature.
- Centrifuge cells for 10 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Continue with Step 2.3.6 from the general protocol.

Δ Note: we recommend performing an optimization run with control cells (GFP-transfected untreated cells) and look at the GFP signal. You might need to vary incubation times and paraformaldehyde percentages to suit your experimental settings.

3. Assay Procedure – IHC Detection

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Each cell line should be evaluated individually to determine the optimal cell density.

3.1 Deparaffinization and rehydration protocol:

Δ Note: this section describes the preparation of formalin-fixed, paraffin-embedded apoptotic tissue section mounted on glass slides. If using fresh-frozen tissue sections, proceed directly to Step 3.2.1.

3.1.1 Remove paraffin by immersing slides in a Coplin jar containing fresh xylene. Incubate 5 minutes at room temperature.
3.1.2 Repeat previous step in a second Coplin jar containing fresh xylene.
3.1.3 Immerse slides in a Coplin jar containing 100% ethanol and incubate 5 minutes at room temperature.
3.1.4 Re-hydrate slides by sequential 3-minutes/room temperature incubations in Coplin jars containing 100% ethanol, 95% ethanol, 85% ethanol, 70% ethanol, 50% ethanol
3.1.5 Immerse slides in a Coplin jar containing 0.85% NaCl and incubate 5 minutes at RT.
3.1.6 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at RT.
3.1.7 Proceed to Step 3.2.3

3.2 Tissue section preparation:

3.2.1 FOR FRESH-FROZEN TISSUE SECTIONS ONLY: fix slides by immersing them in a Coplin jar containing fresh 4% formaldehyde/PBS (w/v). Incubate for 15 minutes at room temperature.
3.2.2 Wash slides by immersing them in a Coplin jar containing PBS and incubate 5 minutes at room temperature.
3.2.3 Repeat washing step in a new Coplin jar containing fresh PBS. Allow liquid to drain thoroughly and place slides on a flat surface.
3.2.4 Prepare 1 mL of 20 µg/mL Proteinase K solution (2 µL Proteinase K 10 mg/mL + 998 µL Tris-HCl pH 8.0 + 50 mM EDTA).
3.2.5 Cover each tissue section with 100 µL and incubate 5 minutes at room temperature.
3.2.6 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at room temperature.
3.2.7 Transfer slides to a Coplin jar containing 4% formaldehyde/PBS and incubate 5 minutes at room temperature.
3.2.8 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at room temperature.

3.3 Label cells:

3.3.1 Remove slides from PBS and tap gently to remove excess liquid. Cover section with 100 µL of Wash Buffer.
3.3.2 Using forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid and incubate 5 minutes at room temperature. Remove plastic coverslip and gently tap the slides to remove excess liquid.
3.3.3 Repeat Step 3.3.2 one more time. Carefully blot dry around the edges with tissue paper.
3.3.4 Cover slides with 50 µL of the DNA Labeling Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
TdT Reaction Buffer	10	100
TdT Enzyme	0.75	7.5
Br-dUTP	8	80
ddH_2O	32.25	322.5
TOTAL VOLUME	51	510

3.3.5 Using forceps, gently place a piece of plastic coverslip on top pf the cells to evenly spread the liquid.
3.3.6 Place slides in a dark humidified 37°C incubator for 1 hour.
Δ Note: ensure high humidity by placing wet paper towels in the bottom of the dry incubator.
3.3.7 Remove the plastic coverslips with forceps. Rinse slides in a fresh Coplin jar filled with PBS for 5 minutes.
3.3.8 Repeat Step 3.3.7 one more time. Blot dry around the edges with tissue paper.
3.3.9 Cover slides with 100 µL of Antibody Solution, prepared as described below:

Component	1 test (µL)	10 tests (µL)
Anti-BrdU-Red antibody	5	50
Rinse Buffer	95	950

3.3.10 Using forceps, place a piece of plastic coverslip on top of the cells to evenly spread the liquid. Incubate slides in the Antibody Solution in the dark for 30 minutes at RT.

3.4 Detection by fluorescence microscopy:

3.4.1 Wash cells by transferring slides to a fresh Coplin jar filled with ddH_2O and incubate for 5 minutes at room temperature.
3.4.2 (Optional DNA counterstaining) Add 100 µL of 7-AAD/RNase A Staining Buffer.
Δ Note: other DNA stains such PI or DRAQ5™ can be used.
3.4.3 Using forceps, place a piece of plastic coverslip on top of the cells to evenly spread the liquid. Incubate slides in the Staining Buffer in the dark for 30 minutes at RT.
3.4.4 Wash cells by transferring slides to a fresh Coplin jar filled with ddH_2O and incubate for 5 minutes at room temperature.
3.4.5 Repeat previous washing step.

3.4.6(Optional) Add a drop of anti-fading solution and cover the treated portion of the slide with a glass coverslip.
3.4.7(Optional) Seal the edges of the coverslip with rubber cement or clear nail polish.
3.4.8Analyze cells by fluorescence microscopy at Ex/Em = 488/576 nm (BrU-Red) and Ex/Em = .488/655 nm (if using 7-AAD).
Δ Note: View slides as soon as possible. Analyze cells within 3 hours of staining.

Data Analysis:

FOR FLOW CYTOMETRY

The following are general guidelines. Specific methods of analysis will vary with different flow cytometer analysis programs.

- Establish appropriate FSC vs SSC gates to exclude debris and cell aggregates.
- Collect BrdU-Red and 7-AAD fluorescence in the appropriate channels (typically, FL2 and FL3 respectively).
- Using fluorescence intensity, determine fold change between control and treated cells.

FOR FLUORESCENCE MICROSCOPY

- We recommend acquiring several images per well.
- We recommend data analysis after coding and mixing images to ensure unbiased results.
- For manual analysis, if you do not have a specific software installed in your microscope, you can download ImageJ, an open source image processing designed for scientific multidimensional images by the National Institute of Health (NIH).

FAQs

Q. Can you give me any tips to perform this assay successfully?

If using adherent cells, cells remaining in the supernatant have a higher probability of being apoptotic than do the cells adhering to the bottom of the plate or flask. Save cells in the supernatant for the assay prior to the trypsinization step.
Cell fixation using a DNA crosslinking chemical fixative is an important step in analyzing apoptosis. Unfixed cells may lose smaller fragments of DNA that have not been chemically fixed inside the cell during the washing steps.
To minimize loss during the assay, restrict the assay to the use of a single 12 x 75 mm test tube. If polystyrene plastic test tubes are used, an electrostatic charge can build up on the sides of the tube. Cells will adhere to the side of the tube and the sequential use of multiple tubes can result in significant cell loss during the assay.
Occasionally a mirror image population of cells at lower intensity is observed in the flow cytometry dual parameter display. The population appears because during the DNA labeling reaction some cells have become stuck to the side of the test tube and are not fully exposed to the reaction solution. This issue can be overcome by washing all the cells from side of the tube and properly resuspend the cells at the beginning of the labeling reaction.

Q. Can I perform a staining for detecting surface marker by flow at the same time?

Staining of proteins such as cellular surface antigens can be accomplished by first incubating the cells with the fluorescent labeled antibody and then using a commercially available fixative and permeabilization solution to rapidly fix and permeabilize the cells in preparation for the assay.

Q. Can I do dual staining for microscopy assay?

Yes, it is possible. It is preferable to follow these recommendations:
For the second target, use an antibody that works best with proteinase K retrieval. We recommend that you run a control test with the same sample and antibody for the other target and see if proteinase K can retrieve the antigen.
Use a fluorophore-based detection system for the second target as opposed to an HRP-based approach.
Perform the BrdU-Red staining (TUNEL staining) before adding the second antibody.
Stain samples with individual targets only as controls.

Q. We used this kit and did not observe any signal under the recommended settings (Ex/Em = 488/576). However, we were able to observe signal after changing the excitation to 555 nm (emission remained the same). Do you have any insights as to why this may be happening, and would you expect the signal they observed to be correct for this kit?

Actually, this is absolutely correct (except that they couldn't excite at 488 is a little troubling) because the Red- anti-BrdU actually has "optimal" excitation at 544 nm. However, we have found that it still excites at 488 nm wavelength, which allows it to use it on a typical flow cytometer and especially on a single laser system such as the BD FACScan, for which it was originally developed. The emission should not change. The same is true for the counterstain used in the kit. It excites very well at 488 (standard flow cytometers) but its optimal excitation is at 546 nm and emission at 647 nm.

Q. What is the spectrum of excitation and emission of the BrdU-Red?

The BrdU-Red monoclonal antibody emits fluorescence at 576nm when excited at 488nm. The wavelengths can shift +/-10 to 20nm based on the instrument.

Q. Can this assay differentiate between necrosis/apoptosis?

TUNEL assay is positive for both necrotic and apoptotic cells and hence cannot distinguish. You can run DNA gel to differentiate between them- while necrotic cells will show a smear due to non-specific cleavage, apoptotic cells has activated endonucleases that cleaves the DNA into fragments of approximately 180-200bp that looks like a ladder on the DNA gel.

Q. Would you please provide any protocol regarding how to adhere the fixed cells (Positive Control provided in the kit) or unfixed cells on the slide?

For fixed cells, as far as we know, you have to use cytospin to put them on the slide for microscopy. Otherwise, you may go with flow cytometry as the detection method.
For non-fixed cells, you can use poly-lysine coated slides/coverslips. Poly-lysine will not work with fixed cells as it based on the interaction between positively charged poly-lysine and negatively charged cells or proteins and in fixed cells, this interaction does not work. Please use a standard protocol for coating the slides/coverslips with poly-lysine.